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# Studies of Antigens for Complement Fixation and Gel Diffusion Tests in the Diagnosis of Infections Caused by *Brucella ovis* and Other *Brucella*

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Sonically treated and saline-extracted antigens of *Brucella ovis*, *B. canis*, *B. abortus*, and *B. melitensis* were compared in gel diffusion, complement fixation, and serum absorption tests. All the sonically extracted antigens showed cross-reactions with sera from animals infected or immunized with these species, whereas the saline-extracted antigens were specific for the surface of the rough or smooth colonial phase of the species or strain. The saline-extracted antigens of *B. ovis* and *B. melitensis* were both eluted as a single peak in the void volume by Sephadex G-200 column chromatography, in gel diffusion had staining characteristics of lipoproteins, but in immunoelectrophoresis showed distinct mobility patterns. Serological activity for both gel diffusion and complement fixation tests was demonstrated in the immunoglobulin G-containing fraction of sera taken from sheep 12 to 412 days after infection with *B. ovis*. The gel diffusion test with saline extract of *B. ovis* is as sensitive as the complement fixation test for the diagnosis of ram epididymitis and is more practical.

Myers and Siniuk (16) described a simple gel diffusion technique for the diagnosis of ram epididymitis which correlated very well with results obtained with the complement fixation test. A soluble antigen obtained by ultrasonic treatment of washed *Brucella ovis* cells (3) was used for both tests. In view of the gel diffusion studies of Diaz et al. (11, 12) showing extensive cross-reactions between the sonically treated antigens of *B. ovis*, *B. melitensis*, *B. abortus*, and *B. canis*, the specificity of this antigen for the diagnosis of ram epididymitis was investigated. Sonically treated and saline-extracted antigens of the four species of *Brucella* were compared in gel diffusion, immunoelectrophoresis, and complement fixation tests. The sonically treated antigens showed cross-reactions with sera from animals infected or immunized with other *Brucella* species. The saline-extracted antigens reacted with either anti-rough phase brucella sera or anti-smooth phase brucella sera, but species-specific antigens were not detected. The saline-extracted antigen of *B. ovis* was as sensitive for the diagnosis of ram epididymitis as the sonically treated antigen in both gel diffusion and complement fixation tests, and did not show cross-reactions with sera from smooth *Brucella* infections. It had the additional advantage of not being anti-complementary.

Serological activity was restricted to the immunoglobulin G (IgG) fractions of sera taken from rams 12 to 412 days after infection with *B. ovis* in both the gel diffusion and the complement fixation tests.

## MATERIALS AND METHODS

**Bacterial cultures.** The cultures employed were *B. ovis*, of rough colonial morphology, a strain isolated by Myers and Siniuk (16); *B. melitensis*, of smooth colonial morphology, strain 16 M (FAO/WHO reference strain), strain H38 (a virulent strain), strain Rev. 1 (an attenuated vaccine strain); *B. abortus*, strain 544 of smooth colonial morphology (FAO/WHO reference strain), strain 45/20 of rough colonial morphology; *B. canis* strain RM 666 of rough colonial morphology.

**Antigenic preparations.** Sonically treated antigens of *B. ovis* and *B. canis* were prepared as described previously (16). *B. melitensis* 16 M and *B. abortus* 45/20 were grown on Trypticase soy agar

without serum, and they were not centrifuged prior to heat-killing at 80 C for 1 hr. They were subjected to sonic vibration in a Raytheon 10-kc magneticostriction oscillator for 20 min and centrifuged. The supernatant fluid was frozen at −20 C and centrifuged again after thawing to remove the lipoid material. The clear supernatant fluid was dialyzed against phosphate-buffered saline, *p*H 7.4, and stored in small tubes at −20 C or lyophilized.

Saline extracts of *B. ovis*, *B. canis*, and *B. melitensis* strain Rev. 1 were prepared by treating freshly harvested buffered saline suspensions of these organisms for 2 hr in an 80 C waterbath. After centrifugation the supernatant fluid was treated in the same manner as that obtained with the sonically extracted antigens.

Antigens were concentrated by means of Carbowax and fractionated in 2-ml amounts through columns of Sephadex G-200 (2 by 90 cm), equilibrated with 0.125 M phosphate buffer (*p*H 7.0) containing 0.02% sodium azide. The optical density of the column eluates was determined spectrophotometrically at 280 nm and fractions were selected as shown in Fig. 2. Fractions from the supernatant antigen were examined directly by gel diffusion and those from the sonic extract were pooled and concentrated five times by means of Carbowax before being examined by gel diffusion.

**Sera.** Sera were obtained from goats infected with *B. melitensis* H 38 or *B. abortus* 544, and from goats vaccinated with living Rev. 1, or killed adjuvant vaccines prepared from *B. abortus* rought 45/20, *B. abortus* smooth 544, or *B. melitensis* H 38 (G. G. Alton, L. M. Jones, C. Garcia-Carrillo, and A. Trenchi, Amer. J. Vet. Res., *in press*). Rabbits were hyperimmunized by intraperitoneal injection of killed *B. canis* without adjuvant.

Selected sheep and goat sera (see Table 4) were fractionated by gel filtration through Sephadex G-200 as described previously (V. M. Varela-Diaz, L. M. Jones, amd M. V. Perez-Esandi, Amer. J. Vet. Res., *in press*).

Selected sera were absorbed with antigens in the following manner. The soluble antigens were freeze-dried in 2-ml amounts, and 0.7 ml of serum was added to the dried antigen. For absorption with whole-cell antigens, a large loopful of growth from a 48-hr culture was suspended in 0.7 ml of serum. The sera containing absorbing antigens were allowed to stand at room temperature for 1 hr and placed directly into the agar wells for the gel diffusion tests. Overnight absorption at 5 C did not remove more precipitins.

**Serological tests.** In the microslide gel diffusion test, an agar cutter prepared according to the specifications described by Myers and Siniuk (16) was employed for most of the examinations. Other patterns with round wells only were also employed. The gel consisted of 1.25% agar (Difco) in *p*H 7.2 buffered saline with 1:10,000 Merthiolate added as a preservative.

Immunoelectrophoresis was carried out on LKB immunoelectrophoresis equipment in *p*H 8.2 buffer gel by the method of Scheidegger (18).

Immunoelectrophoresis and gel diffusion slides were examined before and after staining by the methods described by Crowle (9). Some slides were stained first with Sudan black for an indication of the presence of lipoproteins, restained with amido schwarz (9), and photographed.

The complement fixation test for *B. ovis* infections was described previously (16). Dilutions of sera were employed to compare antigens.

Titers to smooth *Brucella* antigen in the tube agglutination, mercaptoethanol, and complement fixation tests were obtained by methods employed by Jones et al. (L. M. Jones, C. Garcia-Carrillo, and G. G. Alton, Amer. J. Vet. Res., *in press*).

## RESULTS

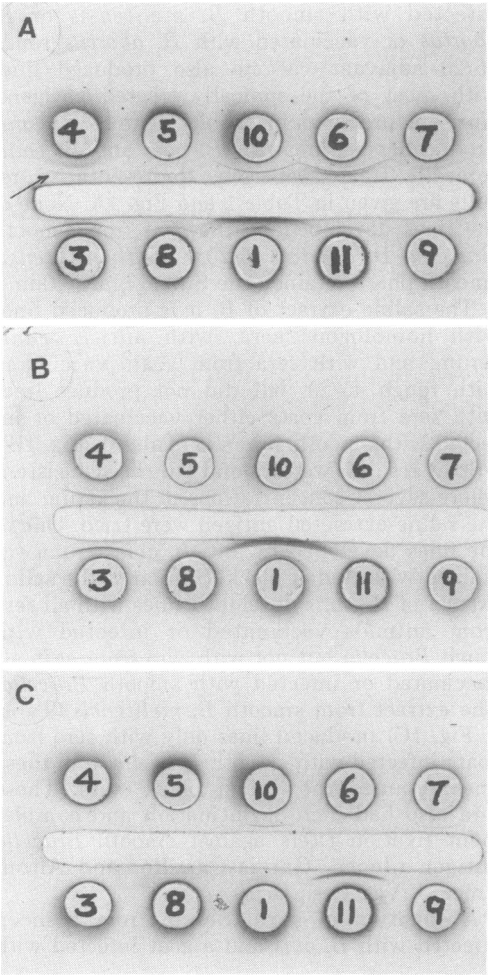
**Sonically treated and saline-extracted antigens in gel diffusion tests.** Sera from sheep infected with *B. ovis* produced precipitin bands in gel diffusion, with sonically treated antigens from *B. ovis*, *B. melitensis*, *B. abortus* 45/20, and *B. canis*. Sera from goats infected with smooth *B. melitensis* or *B. abortus* or vaccinated with *B. abortus* rough 45/20 adjuvant vaccine also produced lines with most of the sonically treated antigens. Antisera prepared in rabbits against *B. canis* produced one line with *B. ovis* and *B. canis* sonically treated antigen. Representative results are given in Table 1 and Fig. 1A. None of the lines developed by the sera from smooth *Brucella*, *B. abortus* 45/20, and *B. melitensis* smooth phase retained the Sudan black stain.

The saline extract of *B. ovis* produced lines with homologous sera, with anti-*B. canis* serum, and with sera from goats vaccinated with rough 45/20, but did not produce lines with sera from goats either vaccinated or infected with smooth *Brucella* (Table 1, Fig. 1B). These results were found to be consistent where several concentrations of the serum and the saline-extracted antigen were tried. One of the lines developed by anti-*B. ovis* serum was stained with Sudan black. Similarly, the saline extract of *B. canis* developed lines with all sera from animals vaccinated or infected with rough *Brucella* but not with sera from animals vaccinated or infected with smooth *Brucella*. The extract from smooth *B. melitensis* (Table 1, Fig. 1C) produced lines only with sera from goats infected with smooth *Brucella*, and these lines retained the Sudan black stain. These sera also had high agglutination and complement fixation titers against smooth *Brucella* antigen (Jones, Garcia-Carrillo, and Alton, Amer. J. Vet. Res., *in press*).

**Absorption of sera.** Serum from a sheep infected with *B. ovis* and a goat infected with

TABLE 1. *Number of precipitin lines obtained in gel diffusion tests with various Brucella antigens and antisera*

| Serum no. | Animal | Inoculum | Sonically treated antigens | | | | Saline extract antigens | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *B. ovis* | *B. canis* | *B. abortus* rough | *B. melitensis* smooth | *B. ovis* | *B. canis* | *B. melitensis* |
| 1 | Sheep | *B. ovis*, living | 4 | 2 | 3 | 3 | 2 | 2 | 0 |
| 2 | Sheep | *B. ovis*, living | 3 | 1 | 2 | 2 | 1 | 1 | 0 |
| 3 | Sheep | *B. ovis*, living | 2 | 2 | 1 | 1 | 2 | 1 | 0 |
| 4 | Sheep | *B. ovis*, living | 3 | 2 | 3 | 1 | 2 | 2 | 0 |
| 5 | Rabbit | *B. canis*, killed | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| 6 | Goat | *B. abortus*, rough, killed with adjuvant | 2 | 2 | 2 | 2 | 1 | 1 | 0 |
| 7 | Goat | *B. abortus*, living | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| 8 | Goat | *B. abortus*, living | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| 9 | Goat | *B. melitensis*, living | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| 10 | Goat | *B. melitensis*, living | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| 11 | Goat | *B. melitensis*, killed with adjuvant | 2 | 0 | 1 | 2 | 0 | 0 | 1 |




*B. melitensis* was absorbed with different antigens and then tested in gel diffusion using these antigens (Table 2). Whole-cell antigens did not remove precipitins for the sonically treated antigens, but they did remove the precipitins for the homologous saline-extracted antigen, i.e., *B. ovis* absorbed the precipitin for *B. ovis* saline extract from anti-*B. ovis* serum; *B. melitensis* cells absorbed the precipitins for *B. melitensis* saline extract from anti-*B. melitensis* serum.

Saline extracts did not absorb precipitins for the sonically treated antigens but they did absorb precipitins for the homologous saline-extracted antigen.

The sonically treated antigens absorbed partially or entirely the precipitins for all the sonically treated antigens in all sera, but removed only the homologous precipitins for the homologous saline-extracted antigen.

This suggests that the saline extracts contained mainly surface antigens which were specific for the colonial type of the strain, whereas the sonically treated antigens contained these antigens as well as internal antigens common to both colonial types.

**Fractionation of antigens by Sephadex G-200 chromatography.** In an attempt to obtain a specific *B. ovis* antigen, the sonic and saline extracts were subjected to gel filtration using

FIG. 1. *Immunodiffusion reactions of (A) B. ovis sonically treated antigen, (B) B. ovis extract antigen, and (C) B. melitensis, smooth extract antigen. The numbered wells correspond to animal sera shown in Table 1. The antigens were placed in the center trench.*

TABLE 2. *Sheep and goat sera absorbed with various antigens and tested in gel diffusion*

| Serum | Antigen for absorption | No. of precipitin lines with: | | | | |
|---|---|---|---|---|---|---|
| | | Sonically treated antigens | | | Saline extract antigens | |
| | | *B. ovis* | *B. abortus* rough | *B. melitensis* | *B. ovis* | *B. melitensis* |
| Sheep infected with *B. ovis* | None | 2 | 2 | 2 | 1 | 0 |
| | Whole cells *B. ovis* | 2 | 2 | 2 | 0 | 0 |
| | Whole cells *B. abortus*, rough | 2 | 2 | 2 | 1 | 0 |
| | Whole cells *B. melitensis* | 2 | 2 | 2 | 1 | 0 |
| | Sonic extract *B. ovis* | 0 | 1 | 1 | 0 | 0 |
| | Sonic extract *B. abortus*, rough | 1 | 0 | 0 | 1 | 0 |
| | Sonic extract *B. melitensis* | 1 | 0 | 0 | 1 | 0 |
| | Saline extract *B. ovis* | 2 | 2 | 2 | 0 | 0 |
| | Saline extract *B. melitensis* | 2 | 2 | 2 | 1 | 0 |
| Goat infected with *B. melitensis* | None | 1 | Not tested | 1 | 0 | 1 |
| | Whole cells *B. ovis* | 1 | | 1 | 0 | 1 |
| | Whole cells *B. abortus*, rough | 1 | | 1 | 0 | 1 |
| | Whole cells *B. melitensis* | 1 | | 1 | 0 | 0 |
| | Sonic extract *B. ovis* | 0 | | 0 | 0 | 1 |
| | Sonic extract *B. melitensis* | 0 | | 0 | 0 | 0 |
| | Saline extract *B. ovis* | 1 | | 1 | 0 | 1 |
| | Saline extract *B. melitensis* | 1 | | 1 | 0 | 0 |

Sephadex G-200. A *B. melitensis* saline extract antigen also was fractionated. The elution pattern in all three cases demonstrated the presence of a peak in the exclusion volume of the column (Fig. 2).

Precipitin band formation was observed (Table 3) when Sephadex fraction I from the *B. ovis* sonic extract was tested in gel diffusion with sera from sheep infected with *B. ovis* and sera from animals immunized with other rough strains, but not with sera from goats vaccinated or infected with smooth *Brucella*. Fraction II of the *B. ovis* sonic extract gave positive reactions with the same sera as the whole sonic extract (Table 1), and fractions III and IV were negative.

All six fractions comprising the single peak eluted after Sephadex fractionation of the *B. ovis* saline extract (Table 3) produced bands in gel diffusion with sera from animals vaccinated or infected with rough *Brucella*, but not with sera from animals vaccinated or infected with smooth *Brucella*. The five fractions in the single peak of the *B. melitensis* saline extract reacted only with anti-smooth *Brucella* sera, and these lines stained with Sudan black.

**Immunoelectrophoretic analysis of antigens.** In order to study their specificity and electrophoretic mobility, immunoelectrophoresis of the antigens against anti-*B. ovis* sheep serum and anti-*B. melitensis* goat serum was performed. The *B. ovis* sonic extract (Fig. 3A) revealed a number of bands with anti-*B. ovis* serum and a single line with the anti-*B. melitensis* serum. The line labeled 1 developed by the two sera showed pronounced staining with Sudan black. The *B. ovis* saline extract (Fig. 3B) developed a wide diffuse arc of anodic mobility only with anti-*B. ovis* serum. A similar arc was detected in the slide of *B. ovis* sonically treated antigen (Fig. 3A, line 2).

The *B. melitensis* (Fig. 3C) and 45/20 sonic extracts (Fig. 3D) revealed several lines with anti-*B. melitensis* serum. These lines were fine, clear, and with slight electrophoretic mobility. The saline extract of *B. melitensis* (Fig. 3E) developed a line only with anti-*B. melitensis* serum having different mobility from that formed by the *B. ovis* supernatant fluid with its homologous serum. This line stained with Sudan black, started just outside of the antigen well, and extended towards the cathode. It was not seen in the *B. melitensis* sonically treated antigen.

**Class of immunoglobulin present in sera of sheep infected with B. ovis.** From sera collected at intervals following artificial infection of sheep with *B. ovis* (16), pools of serum were selected for immunoglobulin analysis by filtration through Sephadex G-200. The fractions were examined by the complement fixation test with sonically treated *B. ovis* antigen and

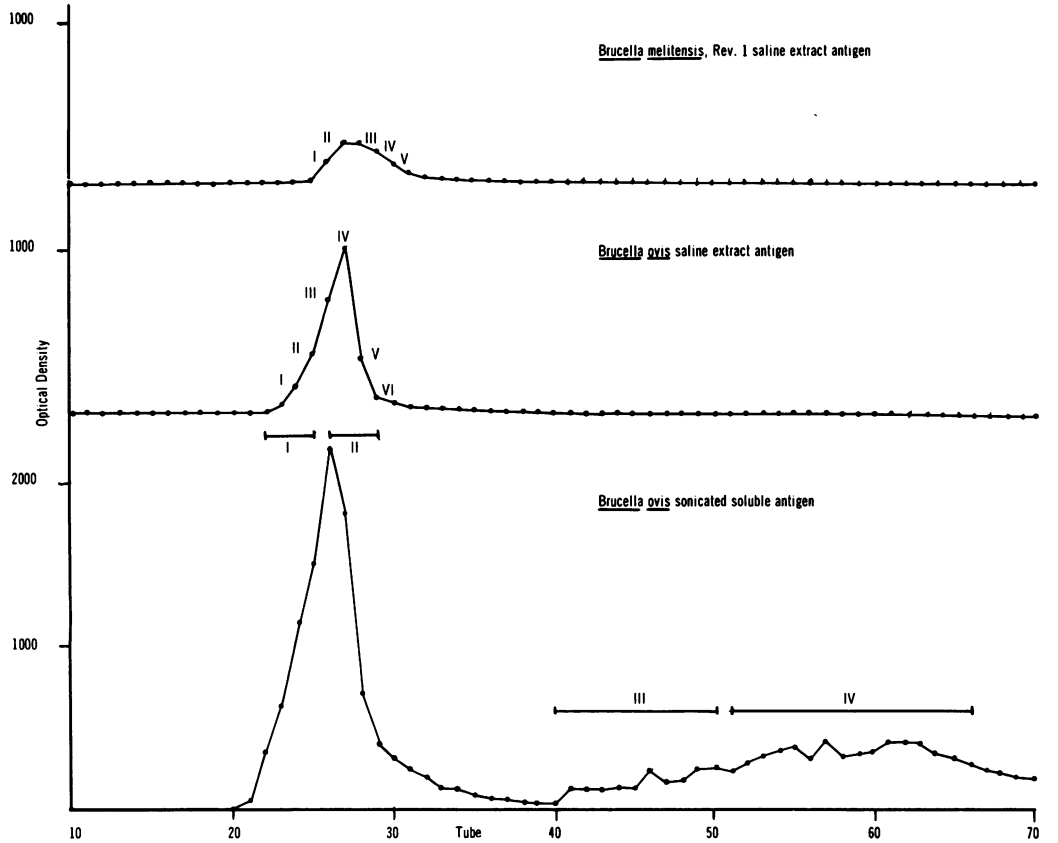



FIG. 2. *Sephadex G-200 elution profiles of B. ovis sonically treated antigen, B. ovis extract antigen, and B. melitensis Rev. 1 saline extract antigen.*

TABLE 3. *Number of precipitin lines obtained in gel diffusion tests with Sephadex fractions of Brucella antigens and various antisera*

| Serum no. | Animal | Inoculum | *B. ovis* sonic extract | | | | *B. ovis* saline extract | | | | | | *B. melitensis* saline extract | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | I | II | III | IV | V | VI | I | II | III | IV | V |
| 1 | Sheep | *B. ovis*, living | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 2 | Sheep | *B. ovis*, living | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 3 | Sheep | *B. ovis*, living | 1 | 2 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 4 | Sheep | *B. ovis*, living | 1 | 2 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 5 | Rabbit | *B. canis*, killed | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 6 | Goat | *B. abortus*, rough, killed with adjuvant | 1 | 2 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 7 | Goat | *B. abortus*, living | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| 8 | Goat | *B. abortus*, living | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| 9 | Goat | *B. melitensis*, living | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| 10 | Goat | *B. melitensis*, living | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| 11 | Goat | *B. melitensis*, killed with adjuvant | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |

by gel diffusion with sonically treated *B. ovis* and *B. melitensis* antigens. The results (Table 4) indicate that both the complement-fixing antibodies and the precipitins were present in the IgG-containing fraction (Sephadex fraction II) from 12 to 412 days after infection. Serum samples taken 26 and 81 days after infection developed lines in gel diffusion with *B. meli-*

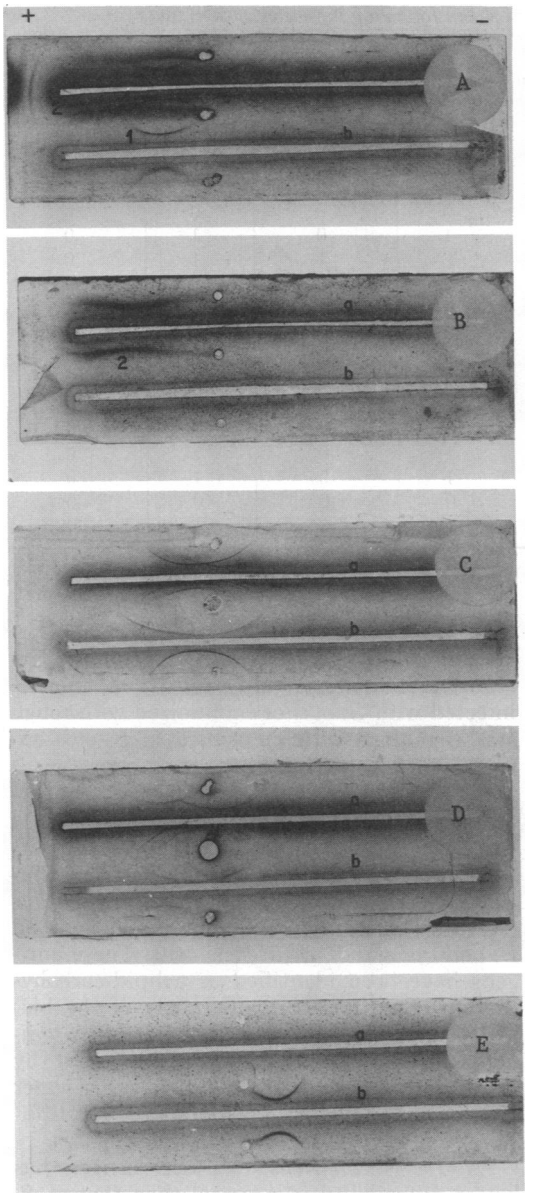



FIG. 3. *Immunoelectrophoresis of (A) B. ovis sonically treated antigen, (B) B. ovis saline-extracted antigen, (C) B. abortus rough 45/20 sonically treated antigen, (D) B. melitensis 16M sonically treated antigen, and (E) B. melitensis Rev. 1 saline-extracted antigen. In each slide the upper trough (a) contains anti-B. ovis sheep serum and the lower trough (b) contains anti-B. melitensis goat serum.*

*tensis* sonically treated antigen, but not with the *B. melitensis* saline extract. The strongest cross-reactions occurred at 81 days (two precipitin bands), at the time of maximum antibody activity as measured by the complement fixation test (titer of 1/1,280). No activity was detected in the immunoglobulin M (IgM)-containing fraction (Sephadex fraction I) even in sera collected 12 days after infection.

**Complement fixation tests with B. ovis antigens.** Myers and Siniuk (16) had found a close agreement between the complement fixation and agar gel diffusion test using the sonically treated *B. ovis* antigen for the diagnosis of ram epididymitis. In the present study the saline extract of *B. ovis* was not found to be anticomplementary, and when compared with the sonically treated antigen, similar titers were obtained. The saline extract antigen had the additional advantage of requiring less complement.

The two *B. ovis* antigens and the *B. melitensis* saline extract were tested in the complement fixation test with the sera shown in Table 1. Both the sonic and saline extracts of *B. ovis* produced complement fixation reactions with rabbit anti-*B. canis* serum and sera from goats vaccinated with rough 45/20, but not with sera from goats vaccinated or infected with smooth *Brucella*. Only the latter sera produced complement fixation reactions with *B. melitensis* saline extract.

**Sensitivity of the gel diffusion test for the diagnosis of smooth Brucella infections.** Two types of antigens were employed in the study of sera from goats vaccinated or infected, or both, with *B. melitensis* and *B. abortus:* (i) soluble surface antigens obtained by saline or phenol extraction of smooth *B. melitensis;* and (ii) sonically treated antigens prepared from *B. ovis* and *B. melitensis*.

Sera containing precipitins to the surface antigens also had agglutinins to smooth antigen (Jones, Garcia-Carrillo, and Alton, Amer. J. Vet. Res., *in press*). Removal of agglutinins by absorption with whole smooth cells removed the precipitins for these antigens. Precipitins to these antigens appeared as early as five days after artificial infection, but they were not always demonstrable in sera with titers to the other tests.

In contrast, the presence of precipitins to the sonically treated antigens was not correlated with the presence of agglutinins to smooth antigens. They were present in sera of animals infected or vaccinated with rough brucella and were most consistently present in sera of goats given adjuvant vaccines. After infection these precipitins appeared later than the precipitins to the surface antigen but they did not always persist as long as the infection.

TABLE 4. *Fractionation of pooled sera from sheep following infection with B. ovis*

| Time after infection (days) | Fraction[a] | Complement fixation with *B. ovis* sonic antigen | No. of lines in gel diffusion | | | |
|---|---|---|---|---|---|---|
| | | | Sonically treated antigen | | Saline extract antigen | |
| | | | *B. ovis* | *B. melitensis* | *B. ovis* | *B. melitensis* |
| 12 | Whole | 160[b] | 2 | 0 | 1 | 0 |
| | S I | 5 | 0 | 0 | 0 | 0 |
| | S II | 40 | 1 | 0 | 1 | 0 |
| 26 | Whole | 160 | 2 | 1 | 1 | 0 |
| | S I | 0 | 0 | 0 | 0 | 0 |
| | S II | 40 | 2 | 1 | 1 | 0 |
| 81 | Whole | 1,280 | 2 | 2 | 1 | 0 |
| | S I | 0 | 0 | 0 | 0 | 0 |
| | S II | 80 | 2 | 2 | 1 | 0 |
| 117 | Whole | 80 | 1 | 0 | 1 | 0 |
| | S I | 0 | 0 | 0 | 0 | 0 |
| | S II | 10 | 1 | 0 | 1 | 0 |
| 412 | Whole | 160 | 1 | 0 | 1 | 0 |
| | S I | 0 | 0 | 0 | 0 | 0 |
| | S II | 5 | 1 | 0 | 1 | 0 |

[a] From Sephadex chromatography.
[b] Reciprocal of titers.

## DISCUSSION

Clapp et al. (6, 8) were the first to describe a complement fixation test for the diagnosis of *B. ovis* infection in sheep. Unlike the original antigen which consisted of a whole-cell suspension, cell-free antigens obtained from boiled or disrupted *B. ovis* cells were more sensitive and not anticomplementary (7). Biberstein and McGowan (3) employed a sonically disintegrated antigen for the complement fixation test, and Myers and Siniuk (16) reported that this antigen was equally sensitive in a simple gel diffusion test which obviated the necessity of the time-consuming and exacting complement fixation technique.

In view of the extensive cross-reactions among the sonically treated antigens of *B. ovis*, *B. canis*, *B. abortus*, and *B. melitensis* demonstrated with hyperimmune rabbit sera (11, 12), the specificity of the gel diffusion test for the diagnosis of ram epididymitis was investigated by using sera from infected animals and from animals immunized with recognized vaccines. In the case of *B. canis*, however, rabbit hyperimmune sera had to be employed because sera from infected dogs was not available.

Precipitin bands were produced by all sera from animals infected or immunized with rough or smooth species of *Brucella* when developed in gel diffusion against the sonically treated *B. ovis* antigen. Similar results were obtained with the sonic extracts of *B. canis*, *B. abortus*, and *B. melitensis*. In complement fixation tests, however, only sera from animals infected with *B. ovis* or immunized with rough strains reacted with the sonicated *B. ovis* antigen.

Smooth and rough *Brucella* show little or no antigenic relationship in agglutination tests using whole-cell antigens (12). The smooth surface antigens contain both the A and M agglutinogens (10) postulated by Wilson and Miles (19) to be present in different proportions on *B. abortus* and *B. melitensis* cells. These antigens have been identified as a lipid-carbohydrate-protein complex with many of the biological properties of the endotoxins of *Enterobacteriaceae* (14) and, when extracted by means of hot phenol-water (1, 14), ether-water (10), or saline (4), produce bands in gel diffusion tests with all anti-smooth brucella sera.

As the *B. melitensis* surface antigen has better diffusibility in agar gel than that of *B. abortus* (4), only the former was used in present study. When extracted with 0.85% saline at 80 C for 2 hr from *B. melitensis* Rev. 1 cells, it produced a single Sudan black-positive band in gel diffusion with anti-*B. abortus* and anti-*B. melitensis* sera, but none with anti-rough *Brucella* sera. The antigen eluted as a single peak in the void volume with Sephadex G-200 column chromatography. In immunoelectrophoresis it showed a wide band beginning just outside the antigen well and extending towards the cathode. This characteristic electrophoretic mobility pattern has been demon-

strated previously by Diaz et al. (10) with an ether-water extract of *B. melitensis*, and by Bhongbhibhat et al. (2) with the first peak eluted in the void volume of Sephadex G-100 column chromatography of a saline extract of *B. melitensis* Rev. 1.

Attempts to isolate a surface antigen with endotoxic properties from rough strains of *B. abortus* (1), *B. ovis* and *B. melitensis* (10), and *B. canis* (12, 13; R. Morisset, C. W. Howe, and W. N. Spink, Bacteriol. Proc., p. 101, 1971) have not been successful. Although a cell-free extract of *B. ovis* has been employed in the complement fixation test (7), it has not been analyzed by immunoelectrophoresis and Sephadex column chromatography previously to our knowledge. In the present study, an antigen specific for the surface of rough brucella cells was obtained in 0.85% saline extract of *B. ovis* cells. This antigen had the same elution characteristics with Sephadex G-200 column chromatography as the smooth surface antigen, was Sudan black-positive in gel diffusion, but had a wide diffuse arc of anodic mobility in immunoelectrophoresis which was in contrast to the cathodic mobility of the smooth surface antigen. Sonically treated *B. ovis* antigen contained the rough surface antigen, demonstrable with immunoelectrophoresis, as well as antigens which cross-reacted with sera from smooth brucella infections.

For the diagnosis of ram epididymitis, the saline extract of *B. ovis* is as sensitive in both the complement fixation and gel diffusion tests as the sonically treated antigen originally employed. It has the advantage of being nonanticomplementary, easier to prepare, and not showing cross-reactions in gel diffusion with sera from smooth infections. This makes it applicable in countries where both *B. ovis* and *B. melitensis* are endemic in sheep.

The gel diffusion method might be practical for the diagnosis of *B. canis* infection in dogs as some difficulties have been encountered in the agglutination test presently employed for that infection (5). It does not appear, however, to be as sensitive as the established methods for the diagnosis of *B. melitensis* and *B. abortus* infections.

Serum samples from sheep infected with *B. ovis* were fractionated by Sephadex G-200 column chromatography. Serological activity with both the complement fixation and gel diffusion tests was demonstrated in the IgG-containing fraction throughout the observation period of 412 days. No activity could be detected in the IgM-containing fraction with either the saline-extracted or sonically treated antigens even in sera obtained 12 days after infection.

A similar restriction of complement fixation activity to the IgG fractions of serum has been found in goats vaccinated or infected with *B. melitensis* (Varela-Diaz, Jones, and Perez-Esandi, Amer. J. Vet. Res., *in press*). In contrast, this activity has also been observed in the IgM fraction of serum from cattle vaccinated or infected with *B. abortus* (17; M. Tiemair, M.S. thesis, Univ. of Wisconsin, 1968). We are not aware of previous immunoglobulin studies in *Brucella*-infected sheep although other workers have shown a shift from IgM to IgG agglutinin activity in sheep vaccinated with smooth *Brucella* antigens (15; V. M. Varela-Diaz, Ph.D. dissertation, Univ. of Pennsylvania, 1970). The specificity of the saline-extracted *B. ovis* antigens for rough *Brucella* strains and the exclusive involvement of IgG in both the complement fixation and double diffusion tests may partially account for the agreement found between these two tests in the diagnosis of ram epididymitis.

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